Evaluation of Genetic Diversity of Bald Cypress (*Taxodium distichum* L. Rich) Growing in Some Governorates of Egypt

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ABSTRACT

Woody species such as trees are ecosystem engineers and landscape modulators as they create resource niches and patches for a whole suite of other organisms dependent upon the development, structural support, decay, and renewal of trees. *Taxodium* is a genus classified in the subfamily *Taxodioideae* of *Cupressaceae*, the cypress family. The wood from *Taxodium* has long been economically valuable due to its resilience, and has been heavily harvested because it is resistant to decay but is also soft, light, very durable, and does not warp easily. Four different localities were selected to survey the Bald cypress (*Taxodium distichum*) samples as follow: El-Beheria Governorate (Nubaria City), Alexandria Governorate (Sabahia farm), Qalyubia Governorate and Giza Governorate (Orman Garden). Five tress were selected from each location except Orman Garden was eight trees. The results showed that there is high significant variation between the collected trees and the data detected that there are in Egypt two types of *Taxodium* the first one is *Taxodium distichum* (L.) Rich found in Alexandria, El-Beheria and Qalyubia Governorates and two different species of *Taxodium* trees in Giza locations grown in dry soil and swamp land, the first five trees were *Taxodium distichum* (Bald cypress) and the other three trees were *Taxodium distichum* var. *imbricarium* (Pond cypress) that grown in swamp land.

Key words: *Taxodium distichum,* morphology, Genetic diversity RAPD and enzymes

INTRODUCTION

Trees are living constituents of many terrestrial and coastal ecosystems, from impenetrable rain forests to open woodlands, from mangrove to riparian, lowland, and subalpine forests (Petit and Hampe, 2002). *Taxodium* is a genus classified in the subfamily *Taxodioideae* of *Cupressaceae*, the cypress family *Taxodiodeae* is a small subfamily consisting of three distinct botanical varieties: *Taxodium* within the southeastern United States and Mexico, *Glyptostrobus* which is native to southeastern China, and *Cryptomeria* which is native to Japan. Variation within the genus *Taxodium* has been interpreted to be either consisting of three separate species, or three varieties within one highly variable species. The three botanical varieties of *Taxodium* are: *Taxodium distichum* (L.) Rich. var. *distichum* known as Bald cypress, *Taxodium distichum* var. *imbricarium* (Nutt.) known as Pond cypress, *Taxodium distichum* var. *mexicana* known as Montezuma cypress (Soltis and Smiley, 1992). Bald cypress in the wild typically grows in a riparian habitat, swamps, or where flooding is common, but it is useful as a landscape tree (Flint, 1994). The tree's landscape assets are remarkable. They commonly grow to reach 60 to 80 feet in height, 3 to 6 feet in diameter, and have a spread of 25 to 35 feet. Bald cypress has a pyramidal profile with symmetrical branching. In general, *Taxodium* are capable of withstanding hurricane conditions, they are easy to grow from seed, easy to transplant, and infestation is seldom a problem (Arnold, 2002; Liberty Hyde Bailey Hortorium, 1976; Lickey and Walker, 2002; Lickey *et al*., 2002; Turner and Watson, 1999; Watson, 1993).

In particular, for forest trees that are outcrossing and largely undomesticated plant species, molecular markers have proven to be invaluable tools with applications in: (1) genetic conservation efforts by identification of genetic diversity hotspots; (2) the assembly of breeding populations in newly developed and advanced breeding programs; (3) the monitoring and characterization of population dynamics and gene flow; (4) the proper delineation of species taxonomy for management issues associated with conservation; (5) assessment of gene flow (pollen contamination) in seed orchards and the authentication of "controlled crossings", the assessment of inbreeding occurrence in breeding programs and studies of mating systems in non-industrial tree species; and (6) genetic fingerprinting in advanced breeding programs for the purpose of quality control to detect misidentified ramets in production and breeding populations (Haines *et al.,* 1994). Genetic markers represent genetic differences between individual organisms or species. All genetic markers occupy specific genomic positions within chromosomes (like genes) called 'loci'. There are three major types of genetic markers: (1) morphological (also 'classical' or 'visible') markers which themselves are phenotypic traits or characters; (2) biochemical markers, which include allelic variants of enzymes

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called isozymes; and (3) DNA (or molecular) markers, which reveal sites of variation in DNA (Jones *et al*., 1981). *Taxodium disticum* L (Fam. Taxodiaceae) has been widely cultivated as an ornamental and timber tree. Therefore, there is a major need to study the genetic background of *Taxodium* species as important genetic resources and also study the genetic variations between the different localities in Egypt by study the morphological characteristic, enzyme activity, calculate the genetic polymorphism with/within *Taxodium distichum* (L.) Rich.

MATERIALS AND METHODS

The present experiments was carried out at the Agricultural Botany Department, Faculty of Agriculture, Saba Basha, Alexandria University, Egypt and Department of timber trees, horticultural research station, Sabahia, Alexandria, Egypt during 2014 to 2016 to study the morphological characteristics, enzyme activity, genetic polymorphism, genetic distance with/within *Taxodium distichum* (L.) Rich based on different morphological, biochemical and molecular markers. Bald cypress is a large, slow-growing but long-lived, deciduous conifer, which frequently reaches 100 to 120 feet in height and 3 to 6 feet in diameter. Its trunk is massive, tapered and buttressed. Four different localities were selected to survey the Bald cypress (*Taxodium distichum*) samples as follow: Alexandria Governorate (Sabhia farm), El-Beheria Governorate (Nubaria City), Qalyubia Governorate (El-kanter El-khaireia Research station farm) and from Orman Garden in Giza Governorate. Five tress were selected from each location except Orman Garden were eight trees.

Morphological studies

Leaves collected for each tree and morphological characters were measured as follow: tree height (meter), diameter (Centimeter), leaves shape, tree age (year).

Biochemical studies (Iso-ezymes electrophoresis)

Agar-starch-polyvinyl pyrolidine (PVP) gel electrophoresis was carried out according to the procedures described by Shaw and Kaen (1967) and Andrews (l981). The extracts were made by grinding from young leaves in a mortar with 10 µl of electrode buffer and centrifuged for 15 sec, a sample of 10 µl of the homogenate was then absorbed onto a small rectangle (about 4mm X 2mm) of filter paper that was placed on the original line of gel plates. The hot liquid gel was poured on glass plates (20 X 30 cm) to produce a smooth surface layer with thickness of 0.8-0.9 mm, and kept at 4 °C until used (El-Metainy *et al*., (1977). 05 gm benzidine and 100µl hydrogen peroxide dissolved in 25 ml distilled water for staining. Incubation was extended for thirty minutes at room temperature and complete darkness. Plates were than distained in distilled water until a clear background of gel plate (Youssef *et al*., 1989).

Random amplified polymorphic DNAs (RAPD-PCR) analysis:

RAPD-PCR has been developed, in which DNA is amplified by the polymerase chain reaction (PCR) using arbitrary short (10 nucleotides) primers according to Williams *et al.* (1990). DNA was extracted following by Murray and Thompson, (1980). RAPD primers (Table 1) were selected from the Operon Kit (Operon Technologies Inc., Alabameda, CA). The polymerase chain reaction mixture (25µl) consisted of 13µl master mix (Promega), *Taq* DNA polymerase; 2µl of genomic DNA, 2 µl primers, 8 µl deionized water. PCR amplification was performed in a Biometra *T1* gradient thermal cycler for 35 cycles after initial denaturation for 5min at 94°C. Each cycle consisted of denaturation at 94°C for 1min; annealing at 36°C for 1min; extension at 72°C for 2min and final extension at 72°C for 5min (Williames, *et al.* 1990). Amplification products were separated on 2% Agarose gels at 100 volts for 1.30 hrs with 1 x TBE buffer. To detect ethidium bromide/DNA complex, Agarose gels were examined on ultraviolet transilluminator (302nm wavelength) and photographed. Using 100pb Plus DNA ladder, ready-to-use (Gene Ruler, Fermentas, and Life Sciences), the lengths of the different DNA fragments were determined. For each sample, the reproducible DNA bands from two runs were scored for their presence or absence.

Table 1. Primers name and their oligonucleotide sequences used in the study

Primer number	Primer code	Sequence (5`-3`)
1	OPC-01	5` TGA TCC CTG G 3`
2	OPC-02	5` CAT CCC CCT G 3`
3	OPA-05	5` AGG GGT CTT G 3`
4	OPA-15	5` TTC CGA ACC C 3`
5	OPB-01	5` GTT TCG CTC C 3`

RAPD's fragments scored as present/absent. Fragment scoring and lane matching performed automatically on digital images of the gels, using Phoretix 1D advanced Version 4.00 (Phoretix International, Newcastle upon Tyne, UK). Clustering methods and similarity coefficients were tested using the program NTSYSpc version 2.10 (Applied Biostatistics, Setauket, New York, USA) (Rohlf, 2000). The percent of polymorphic (P) was calculated using the formula: P=100(p/n) where *p* is the number of polymorphic loci and n is the total number of loci. A locus is polymorphic if the frequency of the allele is less than 0.95.

RESULTS AND DISCUSSIONS

Morphological variations of *Taxodium distichum*

Data in Table 2 showed the morphological description of *Taxodium distichum* trees from the Alexandria governorate for most parameters such as height of trees that ranged from ~16.0 to ~17.8 meter by general average 16.82 meter. The diameter ranged from ~92 to ~120.4 centimeters in general average 130 centimeter. Finally, the tree age was 42 years. The data showed that all the Alexandria trees were Bald cypress. Data in Table 2 showed the difference in morphological parameters in *Taxodium distichum* trees from El-Beheria governorate in relation to tree height ranged from ~11.50 to ~12.8 meter by general average 12.10 meter. The trees diameter ranged from ~102 to ~117 centimeters in general average 109.50 centimeter. Finally, the tree age was 23 years. The data showed that all El-Beheria trees were shaped was soft and green also, all the trees were Bald cypress. The morphological description of *Taxodium distichum* trees from the Giza governorate for height of trees ranged from ~19.30 to ~21.6 meter by general average 20.66 meter. The trees diameter ranged from ~72.6 to ~74.2 centimeter in general average 73.40 centimeter. Finally, the tree age was ~141 years. The data in Table 2 showed that Giza locations have two different species of *Taxodium* trees were grown in soil and swamp land and the first five trees were *Taxodium distichum* (Bald cypress) and the other three trees were *Taxodium distichum* var. *imbricarium* (Pond cypress) that grow in swamp land. Data in Table 2 showed the morphological description of *Taxodium distichum* var. *imbricarium* (Pond cypress) that grow in swamp land from the Giza Governorate such as height of trees that ranged from ~20.80 to ~21.9 meter by general average 21.50 meter. The trees diameter ranged from ~72.6 to ~73.9 centimeter in general average 73.25 centimeter. Finally, the tree age was ~140-145 years and all the trees grow in swamp land. Data in Table 2 described the morphological variations of *Taxodium distichum* trees from the Qalyubia governorate. The highest tree height was ~15.20 meter and the lowest one was 13.09 meter. The trees diameter ranged from ~47.1 to ~54.8 centimeter. Finally, the tree age was ~20 years.

The present results are agreeing with Lickey (2002) concluded that pond cypress and bald cypress can best be described as two varieties of the same species. The same conclusion was reached by Tsumura (1999) used 122 trees representing seven populations of pond cypress and 130 trees representing six populations of bald cypress from Georgia and Florida. Natural selection may have acted similarly upon similar genetic backgrounds in similar habitats which resulted in these similarities in morphology. It is also likely that scenarios, lineage sorting and natural selection, lead to the current morphology of the three *Taxodium* varieties.

Table 2. Description of *Taxodium distichum* trees collected from different Governorate in Egypt

	Tree_1	Tree_2	Tree_3	Tree_4	Tree_5
Alexandria Governorate					
Tree height (meter)	~16.0	~16.7	~17.6	~16.0	~17.8
Diameter (cm)	~100.6	~94.3	~118.5	~92.0	~120.4
Tree age (year)	~42	~42	~42	~42	~42
El-Beheria Governorate					
Tree height (meter)	~12.2	~12.8	~11.8	~ 11.5	~ 12.2
Diameter (Cm)	~ 102	~ 108	~ 105	~ 109	~117
Tree age (year)	~ 23	~ 23	~ 23	~ 23	~ 23
Giza Governorate (soil land)					
Tree height (meter)	~19.3	~21.6	~21.2	~21.6	~19.6
Diameter (Cm)	~72.6	~73.9	~72.6	~74.2	~73.0
Tree age (year)	~140	~143	~141	~142	~140
Giza Governorate (swamp land)					
Tree height (meter)	~21.9	~20.8	~21.9		
Diameter (Cm)	~72.6	~73.9	~72.6		
Tree age (year)	~140-145	~140-145	~140-145		
Qalyubia Governorate					
Tree height (meter)	~13.9	~14.9	~14.2	~15.2	~14.9
Diameter (Cm)	~47.1	~52.9	~53.8	~54.8	~51.3
Tree age (year)	~20-21	~20-21	~20-21	~20-21	~20-21
Soil type	Soil	Soil	Soil	Soil	Soil

Biochemical markers of *Taxodium distichum*

As shown in Figure 1, proxidase isozymes exhibited a wide range of variability among different trees from Alexandria governorate. Two cathodal (Pex. C1 and Pex.C4) were found as common band for all the trees and two bands were different (Pex. C2 and Pex. C3). While the results detected two anodal (Pex.A1 and Pex.A2) bands were recorded in different common band for all trees. The third band appeared in three trees from five. Data detected the total number of amplified bands ranged from 3 to 4 bands with no significant variations between them. Data in Figure 1 for the five different trees from El-Beheria governorate. Two cathodal (Pex. C1 and Pex.C2) were found as common band and two bands were different (Pex. C3 and Pex.C4). Pex. C3 was unique fragment for tree_3. Also, the results detected two anodal (Pex.A1; and Pex.A2) bands for all trees. The third band appeared in three trees from five. Data detected the total number of amplified bands between the five *Taxodium distichum* trees ranged from 3 to 4 bands with no significant variations between them.

Data in Figure 1 for Orman garden (Giza governorate) trees showed high variations for proxidase activity between the first five trees grown in soil land and the other three trees grown in swamp land in Allarroman zoo. One cathodal, (Pex. C1) was found as common band for all the trees. One cathodal (Pex. C4) was found as unique band for the trees 1-5. Two cathodal (Pex. C2 and Pex. C3) were found as unique bands for trees 6, 7 and 8. The same results were observed toward the anodal site that two unique bands were detected (Pex. C2 and Pex.C3) to the last three trees grown in swamp land. Pex. C1 and Pex. C4 were recorded as common band for all trees. Data detected the total number of amplified bands between the eight *Taxodium* species trees used in the current study. The bands number ranged from 4 in *Taxodium distichum* to 8 bands in *Taxodium distichum* var. *imbricarium* (Pond cypress) with high significant variations between them. Data in Figure 1 showed the proxidase activity between the five different trees in Qalubia governorate. Two cathodal (Pex. C1 and Pex.C4) were found as common band for all the trees and two anodal bands were different (Pex. A1 and Pex.A2). Data detected the total number of amplified bands between the five *Taxodium distichum* trees ranged from 3 to 4 bands with no significant variations between them.

Based on the activity of proxidase isozyme as useful tool for genetic classification and calculate the genetic distance between the different species and varieties. Data in Figure 3 detected that all the trees which collected from Alexandria, El-Beheria, Giza and Qalubia from soil land recorded in one cluster by 100% and on the other hand one cluster was observed include the samples from Giza governorate but which grown in swamp land the cluster divided to two groups by 65% similarity. Another marker type that played an important role in assessing genetic diversity in plants was isozymes. Isozymes had a long history in genetic variability studies in forestry, to assess the genetic diversity present within natural forest stands or to determine whether domestication practices had led to a reduction in diversity (El-Kassaby et al., 1994).

C. Molecular studies

The results of primers OPC-01, OPC-02, OPA-05, OPA-15 and OPB-01 are illustrated in Figures 4 Data showed the percentage of polymorphic for *Taxodium* trees collected from Alexandria governorate in Egypt ranged from 25 to 50 % that mean all the collected trees from this location are very similar in all the morphological and genetic back ground that mean this type of trees is one species and namely *Taxodium distichum* or Bald cypress. Data for El-Beheria governorate showed DNA polymorphic ranged from 33 to 46 % and from 25 to 40 % in Qalubia governorate. While, Data from Giza governorate ranged from 38 to 66 % that mean five of the eight collected trees from this location are very different in all the morphological and genetic back ground compression with the other three trees which grown in swamp land that mean this type of trees are two different species based on the different in molecular bases and we can suggest that these sample can divided to two species and namely *Taxodium distichum* (Bald cypress) and *Taxodium distichum* var. *imbricarium* (Pond cypress)

Data in Figure 5 detected the genetic polymorphism and genetic diversity between the collected samples from different locations in Egypt and the overall polymorphism ranged from 33 to 57%. Alexandria, El-Beheria and Qalubia governorates showed almost the same rang of genetic polymorphism, while the samples from Giza governorate showed increase in genetic polymorphism by 23% compression with the average 34% in the three governorates. We can have concluded that the different in genetic polymorphism between three governorates and Giza due to the different in the three trees which grown in swamp land and that classified as *Taxodium distichum* var. *imbricarium* (*Pond cypress*).

The cluster divided to two groups by 34% similarities and the first one includes Alexandria, El-Beheria and Qalubia governorates in one cluster by ~ 100%, while the second one include Giza governorate

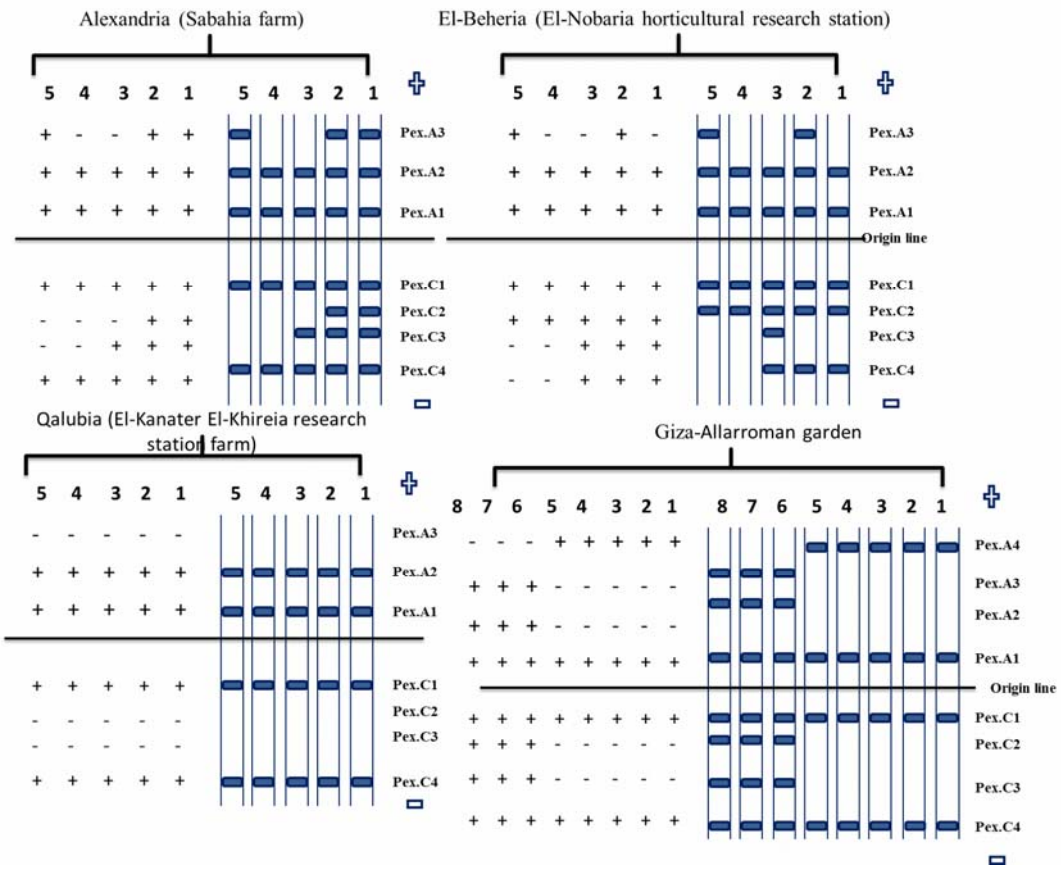

Figure 2. Peroxidase activity and genetic distance between the *Taxodium* trees collected from different localities in Egypt based on proxidase isozyme activity

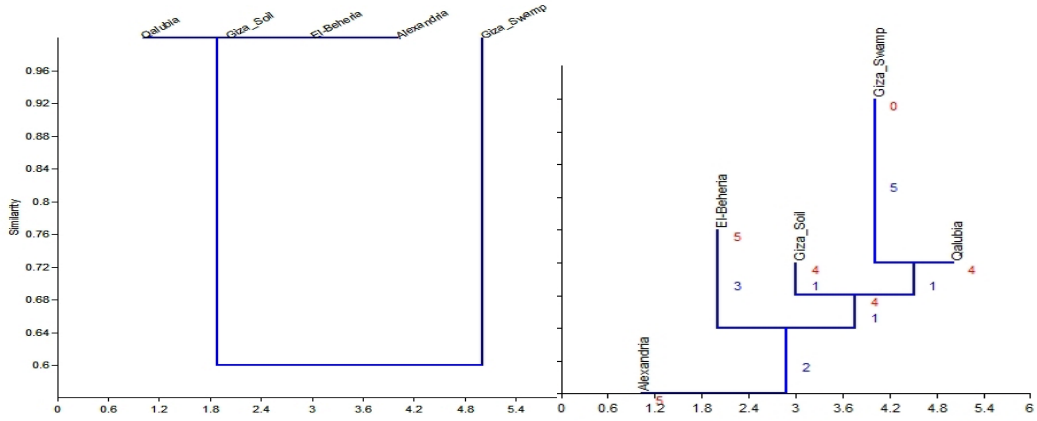

Figure 3. Peroxidase activity and genetic distance between the *Taxodium* trees collected from different localities in Egypt based on proxidase isozyme activity

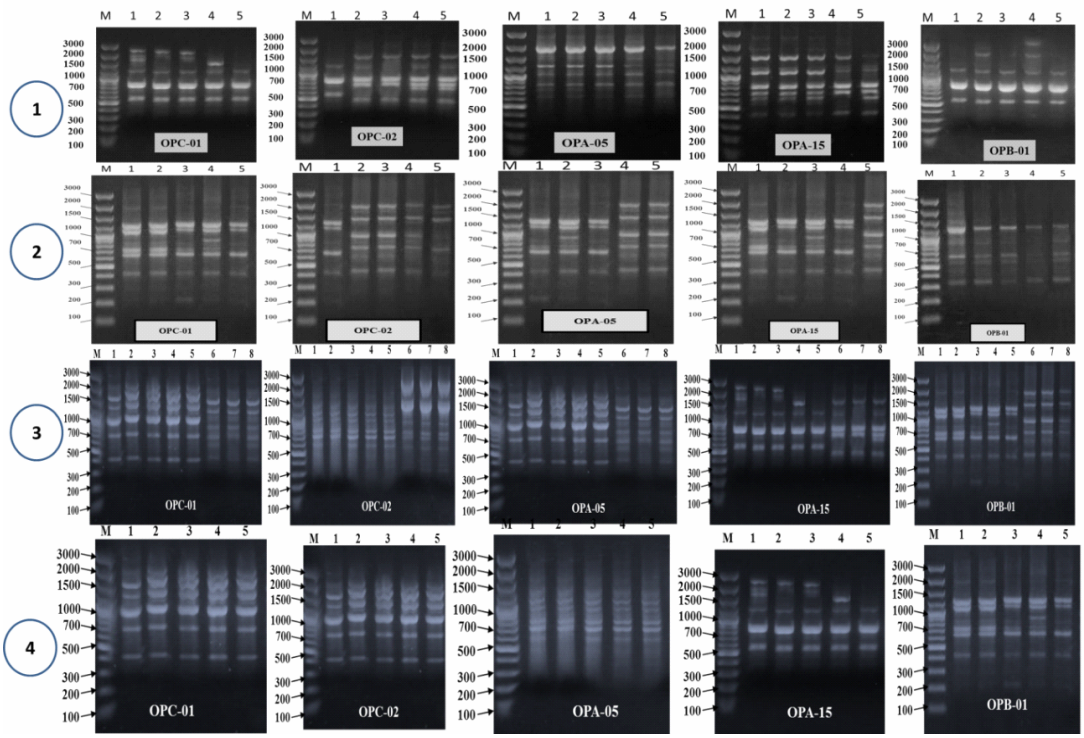

Figure 4. DNA polymorphism of the *Taxodium* trees collected from different governorates in Egypt using randomly amplified polymorphic DNA

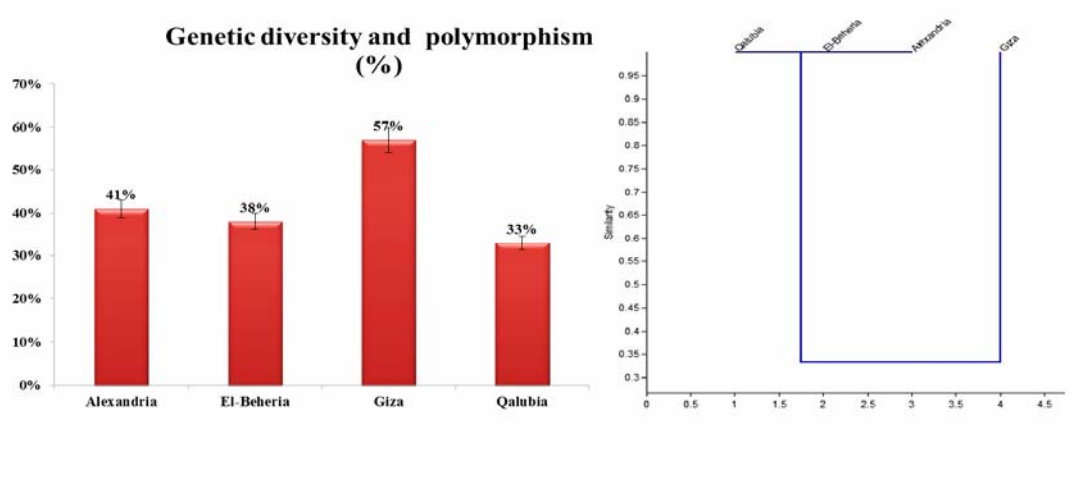

Figure 5. Percentage of Polymorphic and genetic diversity for *Taxodium* trees collected from different localities in Egypt i.e. Alexandria, El-Beheria ،Giza and Qalubia governorates via randomly amplified polymorphic DNA with primers OPC-01, OPC-02, OPA-05, OPA-15 and OPB-01

that mean the same results the three trees which grown in Alorrman Zoo is different species and we detect that *Taxodium distichum* var. *imbricarium* (Pond cypress) based on morphological, biochemical and molecular markers which used in the current study.

Geographically widespread species showed a significantly higher intra-population genetic diversity estimate compared to locally confined species, but the latter showed higher genetic diversity among populations (Hamrick *et al*., 1992). However, the "non-significant" inter-population differentiation sometimes reported in these isozyme studies can mislead the directions of conservation efforts. Our data in the line with Lickey (2002) determined that both bald cypress and pond cypress maintain considerable levels of genetic variation within and among populations. Lickey hypothesizes that pond cypress represents a recently derived taxon which split from the bald cypress during the last glacial cycle, and that either pond cypress represents a recently derived but distinct species which diverged during glaciation, or that pond cypress may represent a variant of bald cypress evolved through adaptations to habitat. He also hypothesized that pond cypress may have become separated from bald cypress during the last glaciation, but through extensive gene flow and introgression, the characteristics of the two taxa have been maintained as clinal extremes. The possibility that pond cypress and bald cypress are two distinct species is unlikely due to their high degree of genetic similarity and the high degree of morphological plasticity of both taxa in response to habitat. The results are agreeing with Zietkiewicz *et al*., (1994) wrote that polymorphism describes the presence of multiple, alternative alleles for a locus within a population, usually expressing different phenotypes. These polymorphisms can be used to determine genetic diversity within and among species. Also, Zietkiewicz *et al*., (1994) provided that ISSR technique provides a genetic fingerprint applicable for taxonomic and phylogenetic comparison as well as for mapping traits and population genetics Yousry El-Kassaby (2014) reviewed that molecular markers have proven to be invaluable tools for assessing plants' genetic resources by improving our understanding with regards to the distribution and the extent of genetic variation within and among species.

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الملخص العربي

تقييم التنوع الوراثى لأشجار التاكسوديوم النامية فى محافظات مختلفة فى مصر

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تعتبر الاشجار الخشبية من اهم المصادر الوراثية النباتية على مستوى العالم لما تتمتع بة من صفات وراثية ممتـازة يمكن الاعتماد عليها فى برامج التربية المختلفة ومن اهـم هذة الاشجار نبات التكسوديوم الذى ينمو بصورة جيدة على مستوى العالم ونظرا للزحف العمرانى على تلك الاشـجار وجب تقييم التنوع الوراثى لها وتحديد اماكن انتشارها وعلية كانت تلك الدراسة بغرض تقيـيم التنـوع الـوراثى لتلـك الاشجار من محافظات مختلفة على مستوى القطر المصرى وقد اختيرت اربع مناطق مختلفة وهى مزرعة بالـصبحية محافظة الاسكندرية ومحافظة القلوبية ومحافظـة الجيـزة ومدينة النوبارية بمحافظـة البحيـرة. اجـرى التوصـيف الوراثى لعدد خمسة اشجار من كل منطقة تضمن ارتفـاع الاشجار وعمرها وقطر الشجرة ووصف عام لحالة الشجرة هذا بالاضافة الى اجراء التحليـل الـوراثى البيوكيميـائى والجزيئيى لنفس الاشجار موضع الدراسة باستخدام دراسـة نشاط انزيم البيروكسيديز وتفاعل البلمرة المستمر اعتمـادا على تفاعل القطع العشوائية. اظهرت النتائج ان هناك تنوع وراثى كبير بين الاشجار موضع الدراسة وبـين المنـاطق المختلفة وقد يعزى هذا التنوع الى البعد الجغرافـى والـى عوامل البيئة المختلفة هذا بالاضافة الى وجود نوعين مـن تلك الاشجار فى مصر والذى يعتبر بمثابـة ثـروة نباتيـة عظيمة وجب الاهتمام بها والحفاظ عليها من الانقراض.